Title

Nuclear PP2A-Cdc55 prevents APC-Cdc20 activation during the spindle assembly checkpoint (SAC)

Authors

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Running title

PP2A inhibits APC during the SAC arrest

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Abstract

Cdc55, a regulatory B-subunit of PP2A complex, is essential for the Spindle Assembly Checkpoint (SAC) in budding yeast, but regulation and molecular targets of PP2A-Cdc55 have not been clearly defined or controversial. Here we show that an important target of Cdc55 in the SAC is Anaphase Promoting Complex (APC) coupled with Cdc20 and that APC-Cdc20 is kept inactive by dephosphorylation by nuclear Cdc55-PP2A when spindle is damaged. By isolating a new class of Cdc55 mutants specifically defective in the SAC and by artificially manipulating nucleocytoplasmic distribution of Cdc55, we further show that nuclear Cdc55 is essential for the SAC. Because Cdc55 binding proteins Zds1/Zds2 inhibit both nuclear accumulation of Cdc55 and SAC activity, we propose that spatial control of PP2A by Zds1-family proteins is important for tight control of SAC and mitotic progression. (133 words)
Introduction

PP2A (protein phosphatase 2A) is a conserved family of serine/threonine phosphatases and has many important roles in mitotic progression in eukaryotes (Shi, 2009). The heterotrimeric PP2A consists with a structural A subunit, a regulatory B subunit and a catalytic C subunit. B subunits bind to the AC heterodimer and regulate both the substrate specificity and the localization of the PP2A complexes. In budding yeast, Cdc55 (B) and Rts1 (B’) have been identified as B-regulatory subunits and bind to the core PP2A in a mutually exclusive manner (Jiang, 2006; Shu et al., 1997; Zhao et al., 1997). Recently, Zds1 (zillion different screens) and its paralogue Zds2 are found to specifically bind to Cdc55-PP2A, but not to Rts1-PP2A (Queralt and Uhlmann, 2008; Wicky et al., 2010; Yasutis et al., 2010) and regulate nucleocytoplasmic distribution of PP2A-Cdc55 complex (Rossio and Yoshida, 2011) adding a new level of complexity.

PP2A-Cdc55 is involved in stress response, polarized growth, meiosis and mitotic progression (Jiang, 2006). In the cytoplasm, PP2A-Cdc55, in complex with Zds1/2, promotes mitotic entry (Rossio and Yoshida, 2011). cdc55Δ and zds1Δ zds2Δ cells exhibit abnormally elongated cell morphology as a consequence of prolonged G2-phase delay (Bi and Pringle, 1996; Healy et al., 1991). The mitotic entry defect of cdc55Δ cells is due to inhibitory phosphorylation of Cdc28 on Tyr 19 by Swe1 kinase because elongated morphology of cdc55Δ is rescued either by deletion of SWE1 (McMillan et al., 1999a; McMillan et al., 1999b; Rossio and Yoshida, 2011; Wang and Burke, 1997; Wicky et al., 2010; Yang et al., 2000) or by introduction of CDC28Y19F mutation.

In the absence of Zds1/2, PP2A-Cdc55 accumulates in the nucleus and prevent mitotic exit (Rossio and Yoshida, 2011). PP2A-Cdc55 has negative roles in mitotic exit
because overexpression of Cdc55 is toxic to the mutants defective in mitotic exit (Wang and Ng, 2006) and because deletion of CDC55 rescues variety of mutants defective in mitotic exit (Clift et al., 2009; Queralt et al., 2006; Wang and Ng, 2006; Yellman and Burke, 2006). The target of PP2A-Cdc55 in mitotic exit is Cdc14 phosphatase, which is essential for mitotic exit in budding yeast (Visintin et al., 1998). Cdc14 is kept inactive by its inhibitor Net1/Cfi1 in the nucleolus (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). Cdc14 release from the nucleolus requires Cdk-dependent phosphorylation of Net1 (Azzam et al., 2004). PP2A-Cdc55 counteracts with Net1 phosphorylation and prevents mitotic exit (Queralt et al., 2006; Queralt and Uhlmann, 2008). Thus, it has been proposed that PP2A-Cdc55 is inactivated at anaphase onset either by nuclear exclusion (Rossio and Yoshida, 2011) or by inhibition of PP2A phosphatase activity (Calabria et al., 2012; Queralt and Uhlmann, 2008).

PP2A-Cdc55 is also an essential regulator of the spindle assembly checkpoint (SAC) (Clift et al., 2009; Evans and Hemmings, 2000; Koren et al., 2004; Minshull et al., 1996; Wang and Burke, 1997; Wang and Ng, 2006; Yellman and Burke, 2006) although the target of PP2A relevant for the SAC has not been demonstrated. cdc55Δ cells, when challenged with microtubule depolymerizing drugs, fail to arrest cell cycle in mitosis and re-enter next round of the cell cycle and die. In cdc55Δ cells, securin Pds1 is degraded in an APC-dependent manner and sister chromatids were separated like other well-characterized SAC mutants (Minshull et al., 1996; Wang and Ng, 2006; Yellman and Burke, 2006) such as mad1Δ or mad2Δ (Alexandru et al., 1999). Interestingly, mitotic cyclins Clb2 and Clb3 remain stable but Cdk1 activity declines and cells exit from mitosis in cdc55Δ (Chirolí et al., 2007; Minshull et al., 1996; Yellman and Burke, 2006).
Recent papers (Wang and Ng, 2006; Yellman and Burke, 2006) proposed that the target of Cdc55 in the SAC is Cdc14, because Cdc14 is precociously released in $cde55\Delta$ and Cdc14 activator Tem1 is dephosphorylated in a manner dependent on Cdc55. However, activation of Cdc14 does not explain why $cde55\Delta$ undergoes Pds1 degradation and precautionary sister chromatid separation upon spindle damages.

Here, we propose that the major target of PP2A-Cdc55 in the SAC is the Anaphase Promoting complex bound to its regulatory subunit Cdc20 (APC-Cdc20). It is known that Cdk1-dependent phosphorylation of at least three APC subunits (Cdc16, Cdc23 and Cdc27) is required for optimal activity of APC-Cdc20 (Rudner and Murray, 2000) but counteracting phosphatase has not been identified. The early finding by Wang and Burke (Wang and Burke, 1997) that $cde55$ deletion rescues the temperature-sensitive growth defect of $cde20\cdot1$ cells, and findings that Tpd3, an A subunit of PP2A can be immunoprecipitated with APC (Boronat and Campbell, 2007; Kornitzer et al., 2001) suggest that PP2A-Cdc55 has a direct role in the regulation of APC-Cdc20 activity.

By isolating novel $cde55$ mutants specifically defective in the SAC, but not the other cell cycle steps, we found that $cde55$ mutation can rescue growth defects of a large variety of APC mutants. We also demonstrate that APC subunits Cdc16 and Cdc27 are hyperphosphorylated in the absence of Cdc55, and unphosphorylatable APC mutants rescued the SAC defects of $cde55\Delta$, supporting that APC-Cdc20 is one of the direct target of Cdc55 in the SAC. In addition, we show evidence that APC-Cdc20 dephosphorylation is taking place within the nucleus. When treated with nocodazole, both Cdc55 and APC are in the nucleus and APC is inhibited. When nuclear accumulation of Cdc55 is perturbed by $cde55\cdot101$ mutation or by Zds1 overexpression, APC-Cdc20 is activated.
and cells become SAC defective. Our results are the first demonstration that APC is a functional target of PP2A-Cdc55 and confirm that regulation of nucleocytoplasmic distribution of PP2A-Cdc55 is important for proper cell cycle control.
Results

Isolation of novel cdc55 alleles specifically defective in the SAC

tmr4 mutants have been identified as recessive-revertant suppressor mutants of temperature sensitive mitotic growth arrest of tom1 mutant (Sasaki et al., 2000; Utsugi et al., 1999). Based on the benomyl sensitivity of tmr4 mutants, we screened plasmids from the genomic library for the ability to complement the benomyl sensitivity of tmr4 mutants. Secondly, we verified whether the plasmids thus inhibited the growth of each tmr4 tom1-2 double mutant at 35°C. By sub-loning and sequence analysis, we identified that tmr4 is allelic to CDC55. We have confirmed the mutant alleles by backcrossing. We have mapped mutation sites of tmr4 mutants (hereafter we named them cdc55-101, -102 and -103) and found that cdc55-101 has a single mutation in Gly 43 to Asp, cdc55-102 has a mutation in Asp 319 to Asn and cdc55-103 has a mutation in Gly 47 to Arg. These mutated residues are highly conserved in PP2A-B55 family from yeast to human (fig. 1A). Based on the recent structural study of PP2A complex, Gly 43 and Gly 47 are located in the β1B sheet and Asp 319 is in the β5C sheet (Li and Virshup, 2002; Xu et al., 2008) (fig. 1B). Because these mutation sites are far away from binding sites to A-C complex and from putative substrates binding region, they are unlikely affecting structural integrity of PP2A complex or enzymatic activity.

It is known that cdc55Δ is defective in mitotic entry and results in abnormally elongated morphology. In a clear contrast to the cdc55Δ, the newly isolated cdc55 mutants did not show elongated morphology or cold sensitivity (fig. 1C), suggesting that these mutants are not defective in mitotic entry. However all three cdc55 mutants showed clear sensitivity to microtubule depolymerizing drug benomyl almost comparable to cdc55Δ (fig. 1C). The benomyl sensitivity of cdc55Δ is associated with misregulation of
catalytic activity of PP2A because overexpression of any of the catalytic subunits, \textit{PPH21}, \textit{PPH22} and \textit{PPH23} partially rescued the benomyl sensitivity (fig. 1D). We also found that overexpression of \textit{SIT4}, a homolog of PP6 phosphatase can rescue the benomyl sensitivity of \textit{cdc55Δ} although the functional relationship between Cdc55 and Sit4 is not clear.

The benomyl sensitivity of \textit{cdc55-101}, \textit{cdc55-102} and \textit{cdc55-103} mutants prompted us to examine the functionality of SAC in these mutants. Indeed, they failed to maintain mitotic arrest in the presence of nocodazole and rebudded and lost viability in similar kinetics to \textit{mad1Δ} (known SAC mutant) (Hoyt et al., 1991; Li and Murray, 1991) and \textit{cdc55Δ} (fig. 2A, B). In addition, we observed that \textit{cdc55-101} mutant failed to maintain sister chromatid cohesion in the nocodazole arrest like \textit{mad1Δ} (fig. 2C) (Hoyt et al., 1991; Stearns et al., 1990; Straight et al., 1996).

Pleiotropic cell cycle defects associated with \textit{cdc55Δ} were one reason that made analysis of Cdc55 function in the SAC difficult. Thus, we took advantage of using our newly isolated \textit{cdc55} mutants uniquely defective in the SAC but not mitotic entry. Because all three \textit{cdc55} mutants showed similar SAC defects we focused our analysis on \textit{cdc55-101}.

To further characterize SAC defects of \textit{cdc55-101} we synchronized yeast cells in G1 phase by mating pheromone and released into the medium containing nocodazole. In wild type strain, securin Pds1, S-phase cyclin Clb5 and mitotic cyclin Clb2 were all stabilized (fig. 2D and S1) due to SAC dependent inhibition of APC-Cdc20 (Visintin et al., 1997). In the SAC defective \textit{mad2Δ} strain, both Pds1 and Clb2 were precociously
degraded (fig. 2D). In addition Clb2 is gradually degraded as consequence of mitotic exit and activation of APC-Cdh1 (Zachariae et al., 1998). In cdc55-101, like mad2Δ, Pds1 and Clb5 were precociously degraded. Consistent with previous reports (Chiroli et al., 2007; Minshull et al., 1996; Yellman and Burke, 2006) we didn’t see clear effect on Clb2 degradation (fig. 2D) suggesting that Cdc55-101 has specific effect on APC-Cdc20 but not APC-Cdh1. We also confirmed that the timing of Pds1 and Clb2 degradation in cdc55-101 is very similar to that of cdc55Δ but distinct from mad2Δ (Fig. 2D). Thus cdc55-101 mutant is completely defective in Cdc55 function in the SAC.

Cdc55 regulates APC-Cdc20

It is known that cdc20-1 temperature sensitivity (ts) is suppressed by cdc55Δ (Wang and Burke, 1997). We found that cdc55-101, cdc55-102 and cdc55-103 also rescued the ts of cdc20-3 (fig. 3A). The suppression of cdc20-3 is not simply due to a SAC defect because neither mad1Δ nor bub2Δ rescued cdc20-3 (fig. 3A). Furthermore, not only cdc20-3, but also several other APC mutants, including cdc16-1, cdc26Δ, apc1-1 were rescued by cdc55-101 mutation (fig. 3B). These genetic data suggest that Cdc55 has specific function in controlling APC-Cdc20 activation or localization.

It is known that activation of APC-Cdc20 requires Cdk-dependent phosphorylation (Rudner et al., 2000; Rudner and Murray, 2000). At least three subunits of APC (Cdc16, Cdc23, Cdc27), are phosphorylated both in vitro and in vivo by Cdk and mutating these phosphorylation sites (Cdc16-6A, Cdc23-A, Cdc27-5A) impairs APC functions in vivo (Rudner and Murray, 2000). Because Cdk1 and PP2A counteracts, we hypothesized that PP2A-Cdc55 inhibits APC-Cdc20 by dephosphorylation. We found that Cdk dependent phosphorylation of Cdc16 and Cdc27 is controlled by Cdc55. Cdc16,
Cdc23 and Cdc27 are known to be phosphorylated by Cdk and migrate slowly in the SDS-PAGE after Cdk-dependent phosphorylation (Rudner and Murray, 2000). Slow migrating species of Cdc16-HA and Cdc27-HA by Western blotting is more abundant in cdc55Δ when cells were arrested by SAC activation in nocodazole (fig. 4A), indicating that PP2A-Cdc55 is required for dephosphorylation of Cdc16 and Cdc27 in this condition. We also found that slow migrating form of Cdc16-HA is accumulated in cdc55-101, similar to cdc55Δ after nocodazole treatment (Fig. 4C) We have also tested Cdc23 in the same condition but were not able to see obvious mobility shift in the cdc55Δ (not shown) most likely due to the fewer numbers of phosphorylation sites on Cdc23 than Cdc16 and Cdc27 (Rudner and Murray, 2000).

To confirm the SAC defect of cdc55Δ is due to hyperphosphorylation of APC, we combined cdc55 deletion with the unphosphorylatable APC mutants (Cdc16-6A, Cdc23-A, Cdc27-5A) and found that the SAC defects of cdc55 is almost completely rescued by these mutations (fig. 4B). This genetic data suggests that the major target of PP2A-Cdc55 in the SAC is the APC.

We also examined localization of an APC subunit Cdc23. As it has been reported, Cdc23-GFP is constitutively in the nucleus and sometimes localized to the spindles (Jaquenoud et al., 2002; Melloy and Holloway, 2004) in both WT and in cdc55Δ (fig. S4). Activation of SAC by addition of nocodazole had no clear effect on general nuclear Cdc23-GFP localization (fig. S2). Thus, phosphorylation of APC subunits is not affecting APC localization. This is consistent with previous data that indicated that localization of Cdc23 is not altered in a strain with all Cdc28 consensus sites mutated in the APC/C subunits Cdc23, Cdc27 and Cdc16 (Melloy and Holloway, 2004).
It is formally possible that APC is not the only target of Cdc55 in the SAC. To examine contribution of Cdc14 release by phosphorylation of Net1 in the SAC defect of cdc55Δ, we have tested if unphosphorylatable Net1 (NET1-6cdk-9myc) (Azzam et al., 2004) can rescue the SAC defects of cdc55Δ. Indeed, NET1-6cdk-9myc partially rescued the SAC defects of cdc55Δ, however NET1-9myc used as a negative control, also rescued the SAC defects of cdc55Δ (fig. S3). This result suggests that 9xmyc tag of Net1 has an unexpected dominant role in delaying mitotic exit and contribution of Cdk dependent phosphorylation of Net1 in the SAC remained unclear.

**Cdc55-101 is cytoplasmic**

Cdc55 localizes to the polarized growth sites as well as cytoplasm and in the nucleus (Gentry and Hallberg, 2002). We have recently shown that Cdc55 exert its specific function in a specific compartment (Rossio and Yoshida, 2011). For examples Cdc55 promotes mitotic entry in the cytoplasm while nuclear Cdc55 delays mitotic exit. It is known that APC-Cdc20 and its substrates are in the nucleus (Jaquenoud et al., 2002; Melloy and Holloway, 2004), we anticipated that nuclear Cdc55 is responsible for APC dephosphorylation. Because Cdc55-101 is competent with mitotic entry (fig. 1C) but defective in the SAC (fig. 2) we suspected that Cdc55-101 was mislocalized from the nucleus.

Cdc55-GFP was localized to both in the nucleus and in the cytoplasm as judged by Nup159-mCherry staining of nuclear envelope (fig. 5A), in contrast Cdc55-101-GFP was excluded from the nucleus throughout the cell cycle (fig. 5A). Loss of nuclear signal was not due to reduced expression or instability of Cdc55-101-GFP protein because Cdc55-101-GFP was expressed to the similar levels to wild type Cdc55-GFP by Western
blotting (fig. 5B). Similar nuclear exclusion was observed with Cdc55-103-GFP (fig. S4). Cdc55-102-GFP was also partially excluded from the nucleus, but the effect was not as strong as Cdc55-101 and Cdc55-103, suggesting that this mutant may have minor effect on localization.

Importantly, when SAC is activated by nocodazole, Cdc55 is found both in the nucleus and in the cytoplasm (fig. 5C). In a sharp contrast, Cdc55-101 is barely accumulated in the nucleus (3.5% of the cells have Cdc55-101 in the nucleus compared to 89% of the control). These results are consistent with our hypothesis that nuclear Cdc55 is responsible for the SAC.

**Overexpression of Zds1 impairs SAC**

To test if nuclear exclusion of Cdc55 results in activation of APC-Cdc20 and in the SAC defect, we examined an effect of Zds1 overexpression. Zds1 forms a stoichiometric complex with PP2A-Cdc55 complex (Queralt and Uhlmann, 2008; Wicky et al., 2010; Yasutis et al., 2010) and promotes cytoplasmic functions of Cdc55 but interferes with nuclear functions of Cdc55 (Rossio and Yoshida, 2011).

We first confirmed that Zds1 is a cytoplasmic protein (Bi and Pringle, 1996; Rossio and Yoshida, 2011) and always excluded from the nucleus by using Nup159-mCherry marker (fig. S5A). We also confirmed that Cdc55 is excluded from the nucleus after ZDS1 overexpression (Rossio and Yoshida, 2011) by using nuclear envelope marker Nup159-mCherry (Fig. S5B).

Temperature sensitive growth defect of cdc20-3 was effectively suppressed by overexpression of ZDS1 (fig. 6A). Thus, Zds1 overexpression caused a similar effect to cdc55-101 mutation or to cdc55Δ.
To assess the effect of ZDS1 overexpression on Cdc55 in the SAC, we used zds1Δc800, which lacks Cdc55-binding domain (CBD) as a negative control. To note, we found highly conserved residues within CBD of fungal Zds1-family proteins and animal cortactin binding proteins CTTNBP2 and CTTNBP2NL (fig. 6B), which are recently identified as PP2A-B55 (PP2A-Striatin) binding proteins (Goudreault et al., 2009) and critical regulator of PP2A-B55 localization to the dendritic spines (Chen et al., 2012).

Overexpression of ZDS1 but not zds1Δc800 caused sensitivity to benomyl (fig. 6C). Cells overexpressing ZDS1 were not able to arrest in nocodazole and rebudded, which is characteristic to SAC mutants, and this effect is dependent on the ability of Zds1 to bind to Cdc55 because overexpression of zds1Δc800 did not cause these defects (fig. 6CE), although Zds1Δc800 was expressed to the similar level to the full length Zds1 (Fig. 6D).

Thus, overexpression of Zds1 has similar effect to loss of Cdc55 and causes SAC defects most likely due to depletion of nuclear Cdc55.

**Nuclear Cdc55 is essential for the SAC**

To further test if nuclear Cdc55 is responsible for the SAC arrest, we took advantage of using engineered cdc55 mutants, which are predominantly either cytoplasmic (cdc55-NES) or nuclear (cdc55-NLS)(Rossio and Yoshida, 2011). We also tested zds1Δzds2Δ mutant where Cdc55 is predominantly nuclear (Rossio and Yoshida, 2011). First, we found that cdc55-NES strain is sensitive to benomyl like cdc55Δ (fig. 7A). Note that cdc55-NES is not simply a loss of function because cdc55-NES has dominant function in the cytoplasm and can induce mitotic entry in zds1Δzds2Δ (Rossio and Yoshida, 2011).
*cdc55–NES* was indeed defective in the SAC. *cdc55-NES* strain rebudded in the presence of nocodazole like *cdc55A* (fig. 7C). In contrast, *zds1Δzds2Δ* was competent in the SAC (fig. 7C). We also confirmed the SAC defects of *cdc55-NES* by FACS analysis of the DNA contents and found that *cdc55-NES* reduplicated its DNA in the presence of nocodazole as *cdc55A* did (fig. 7B). In these assays we have deleted *SWE1* gene to exclude potential G2 phase cell cycle delay by *cdc55* or *zds1 Δzds2 Δ* mutations.

Thus, loss of Cdc55 from the nucleus by *cdc55-NES* caused a similar effect to *cdc55-101* mutation or to *cdc55A* or to *ZDS1* overexpression.
DISCUSSION

Cdc55 inhibits APC-Cdc20

In this study, we have shown that the critical target of Cdc55 in the SAC is APC-Cdc20 because phosphorylation status of APC subunits are controlled by Cdc55 in vivo and the SAC defects of cdc55 is largely rescued by preventing APC phosphorylation.

The targets of Cdc55 in the SAC have long been debated. Since SAC defect is accompanied by mitotic exit, recent studies suggested that Cdc14 activation is a key step prevented by Cdc55 during SAC arrest (Wang and Ng, 2006; Yellman and Burke, 2006). However, we favor APC-Cdc20 activity than Cdc14 activity as a key target of Cdc55 phosphatase in the SAC because Cdc14 activates APC-Cdh1, not APC-Cdc20 (Jaspersen et al., 1999; Visintin et al., 1998; Zachariae et al., 1998).

The fact that APC-Cdc20 activity but not APC-Cdh1 activity is promoted by Cdk1-dependent phosphorylation (Rudner and Murray, 2000) and our data that preferential degradation of APC-Cdc20 substrates Pds1 and Clb5 but not Clb2, a major target of APC-Cdh1, in cdc55 is consistent with APC-Cdc20 being under Cdc55 control.

It is important to mention that Cdc14 activation and subsequent APC-Cdh1 activation is under APC-Cdc20 control. Thus Cdc14 and APC-Cdh1 should be eventually activated in the cdc55 mutants arrested in spindle damages. Unfortunately, we were not able to address direct contribution of Cdc14 inhibition by Cdc55 in the SAC because effects 9xmyc tag on the NET1, not the mutation in the Cdk1-dependent phosphorylation sites of Net1, caused suppression of the SAC defects of cdc55Δ.
Although deletion of NET1 rescues various mitotic exit mutants similar to deletion of CDC55 (Straight et al., 1999; Visintin et al., 1999; Yellman and Burke, 2006), deletion of NET1 does not suppress cdc20-1 (Visintin et al., 1999). Highly unique suppression of APC-Cdc20 function by cdc55 mutations is consistent with our idea that Cdc55 not only regulates Net1 for Cdc14 release, but also APC-Cdc20 for sister chromatid separation.

Roles of PP2A in the regulation of APC and SAC are currently not clear in animal cells because of redundancy of the PP2A subunits (Shi, 2009; Virshup and Shenolikar, 2009) and because of pleiotropic defects associated with PP2A inhibition (Janssens and Goris, 2001). Several studies implicate APC activity is negatively regulated by PP2A in animal cells. In Drosophila, the metaphase-like arrest of mks (Cdc27 homologue) mutant can be suppressed by mutations in the twins/aar (CDC55 homologue) gene, but not by mutations in the SAC genes (Deak et al., 2003). In human, Cdc55 homologue PRB2B regulates phosphorylation status of Cdc27 and affects localization of Cdc27 to the mitotic spindle (Torres et al., 2010). Thus it is highly likely that regulation of APC by PP2A is an evolutionarily highly conserved process.

**Suppressors of tom1 mutant**

We have isolated novel cdc55 mutants as revertant suppressors of tom1-2 mutant. tom1 mutant arrest cell cycle in G2/M phase but substrates and targets of Tom1 important for mitotic progression have not been identified (Sasaki et al., 2000). In contrast to cdc55Δ, which showed abnormally elongated morphology and cold sensitive phenotype in addition to the SAC defect, the only phenotype associated with cdc55-101, -102, -103 was the SAC defect. Because Cdc55-101 protein was expressed to the wild type level and
because mutation sites are not located in the binding surface to A-C subunits, it is unlikely that \textit{cdc55-101} is impaired in PP2A complex assembly or PP2A catalytic activity. The absence of apparent morphological defects in \textit{cdc55-101} also suggests that PP2A activity is not severely impaired. It is formally possible that Cdc55-101 protein has lost interaction with specific substrates such as APC, but we favor the idea that Cdc55-101 is specifically defective in its nuclear localization because the phenotype of \textit{cdc55-101} was very similar to that of \textit{cdc55-NES} strain and that of the cells overexpressing ZDS1.

The targets of Tom1 critical for mitotic progression are not understood yet, but it is most likely APC-Cdc20 activity. Interestingly, in the same screen for \textit{tmr4/cdc55}, \textit{tmr1/cyr1} and \textit{tmr2/sch9} have also been identified (Sasaki T, 2000). Cyr1 encodes an adenylate cyclase and Sch9 encodes a kinase antagonizing PKA pathway. In addition, suppression of PKA pathway either by overexpression of \textit{BCY1}, a regulatory subunit of PKA or by overexpression of \textit{PDE2}, a phosphodiesterase, can also rescue growth defect of \textit{tom1} mutants (Sasaki T, 2000). It is known that PKA activity inhibits APC-Cdc20 activity by phosphorylating Cdc20 (Anghileri et al., 1999; Searle et al., 2004). Because inhibition of PKA activity rescue mitotic defects associated with \textit{tom1} mutation, we suspect that activation of APC-Cdc20 is the key process compromised in \textit{tom1} mutant.

\textbf{Compartmentalized function of PP2A-Cdc55 and its regulation by Zds1}

We showed that both Cdc55 and APC subunits are in the nucleus in nocodazole-arrested cells. Nuclear localization of APC during mitosis is consistent with the fact that Cdc20 as well as its substrates Pds1 and Clb5 are predominantly nuclear (Jaquenou et al., 2002).
Three lines of evidence suggest that stable SAC arrest and inhibition of APC-Cdc20 activity requires PP2A-Cdc55 activity in the nucleus. First, a SAC defective Cdc55-101 mutant protein is excluded from the nucleus. Second, retention of Cdc55 in the cytoplasm by overexpression of ZDS1 impaired SAC. And third, forced exclusion of Cdc55 from the nucleus by adding strong nuclear export signal (cdc55-NES) results in SAC defects. In these cases, temperature sensitive growth defect of cdc20-3 was also rescued.

In the previous study, we reported that Cdc55 localization changes during cell cycle and nuclear Cdc55 signal is high in G1 or small budded cells but is reduced in mitotic cells in a Zds1/2 dependent manner (Rossio and Yoshida, 2011). Exclusion of Cdc55 from the nucleus during mitosis is consistent with our model that nuclear Cdc55 interfere with APC-Cdc20 activity (this study) and with Cdc14 activation (Rossio and Yoshida, 2011) (Fig. 8).

The mechanism by which Zds1/2 exports Cdc55 containing PP2A complex and how interaction between Zds1/2 and Cdc55 is regulated during the cell cycle and upon SAC activation is a very important question. We did not clearly see obvious change in nuclear localization of Cdc55 upon SAC activation (data not shown), suggesting that PP2A-Cdc55 is not specifically responding to spindle damage, rather setting high threshold for Cdk1-dependent activation of APC to prevent hyperactivation of APC-Cdc20.

Although Zds1 is highly conserved in almost all fungal species, no obvious homologue was found in animals or plants. By carefully examining Cdc55 binding domain sequence of Zds1, we found conserved homology in PP2A binding region of
human CTNTBP2NL and CTNTBP2. Importantly, CTNTBP2NL and CTNTB2 specifically bind to regulatory B subunit of PP2A in the region with high homology to CBD of Zds1 and this interaction is essential for PP2A complex to localize to the dendritic spines (Chen et al., 2012). Thus Zds1 and CTNTBP2 are functioning in a similar manner to recruit PP2A complex to specific subcellular components. We propose that spatial control of PP2A complex by Zds1-like regulatory proteins is a widely conserved mechanism to compartmentalize PP2A activity.
**Materials and Methods**

**Yeast genetics**

All yeast strains used in this study were isogenic or congenic to BY4741 (MATα leu2Δ0 his3Δ1 met15Δ0 ura3Δ0, obtained from Thermo Fisher Scientific) or to W303 (Mata, ade2-1, trp1-1, leu2-3,112, his3-11,15, ura3, ssd1 obtained from Thermo Fisher Scientific). Standard yeast genetics was used to generate the strains. Yeast strains are listed in Table S1. PY3295 and SY strains were gifts from D. Pellman (Dana-Farber Cancer Institute, Boston, MA). D. Lew (Duke University, Durham, NC) provided a SWE1 gene knockout plasmid. Gene deletions or modifications were performed with PCR-mediated one-step gene replacement using pFA6a vectors provided by J. Pringle (Stanford University, Stanford, CA) (Longtine et al., 1998) and confirmed by PCR. The LacO/LacI system for monitoring sister chromatid cohesion was a gift from D. Koshland (UC Berkeley, CA).

The ZDS1 plasmid was purchased from the National Bio-Resource Project (NBRP). α-factor was used at 2 µg/ml. Nocodazole was used at 15 µg/ml. Benomyl was used at 12.5 µg/ml or 7.5 µg/ml or 15 µg/ml as indicated in the legends. For galactose induction, 2% of galactose was added to the medium.

**Biochemistry**

Protein extracts were prepared by trichloroacetic acid (TCA) precipitation as previously described (Piatti et al., 1996). Mouse anti-HA (16B12 Roche and Covance), Mouse anti-GFP antibody (Millipore), Mouse anti-Myc (9E10, Wako) and anti-PSTAIRE (sc-53, Santa Cruz) were purchased from the commercial source. HRP-conjugated secondary
antibodies were obtained from Millipore, and proteins were detected by an enhanced chemiluminescence system (ECL Prime; GE Healthcare).

**FACS analysis**

For FACS (Fluorescence-Activated Cell Sorter) analysis cells were collected by centrifugation and then fixed in 70% Ethanol. Cells were then washed once with 1 ml of Tris/HCL 50 mM pH 7.5 and the pellet was then resuspended in 0.5 ml of Tris/HCL 50 mM pH 7.5 containing 1 mg/ml RNAse. After incubation over night at 37°C, cells were collected by centrifugation cells were then washed once with 1 ml of FACS Buffer (200 mM Tris/HCL pH 7.5, 200 mM NaCl, 78 mM MgCl₂) and resuspended in the same buffer containing 50 μg/ml Propidium Iodide.

**Viability assay**

Logarithmically growing cells are collected and resuspended in YPD containing nocodazole (15μg/ml). Two hundred cells are plated on a YPD plate at each time points after nocodazole addition. Once colonies have formed, viability was calculated by dividing the number of colonies formed at the different time points by the number of colonies formed at time 0. The rate of death in the presence of nocodazole is an excellent indicator of checkpoint deficiency (Hoyt et al., 1991).

**Rebudding and sister chromatid separation assays**

Wild-type cells arrest in response to SAC activation as large budded cells in mitosis. The checkpoint mutants continue through the cell cycle and they eventually pass through the subsequent G1 generating a new bud. Logarithmically growing cells are treated with nocodazole (15μg/ml). At each indicated time point cells are fixed in 70% ethanol at room temperature. Cells are then washed two times in PBS, vortexed vigorously,
examined by bright-field microscopy and categorized into G1 (unbudded), dumbell (large budded) and rebudded cells (more than one bud).

Sister chromatid separation was monitored by visualizing the right arm of chromosome XV using the LacO/green fluorescent protein (GFP)-LacI system (Straight et al., 1996).

**Fluorescence microscopy**

Fluorescence images were acquired with a fluorescence microscope (Eclipse E600; Nikon) equipped with a charge-coupled device camera (DC350F; Andor) with 100Å~, NA of 1.45, or 60Å~, NA 1.4, oil objectives. The images were captured and analyzed with NIS-Elements software (Nikon), and the figures were processed and assembled in Photoshop or ImageJ (Adobe). Fluorescence image of Zds1-GFP in the presence of Nup159-mcherry (Fig.S2) were acquired with a Spinning Disk Confocal Microscope (Zeiss AxioObserver) with PLAN APOCHROMAT 63x/1.4 oil M27 with DIC prism. The image were captured and analyzed with SlideBOOKTM software and the figures were processed and assembled in ImageJ or Photoshop (Adobe).
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Abbreviations

APC, anaphase promoting complex; CBD, Cdc55-binding domain; NES, nuclear export signal; PP2A, type 2A protein phosphatase; SAC, spindle assembly checkpoint;
References


Deak, P., M. Donaldson, and D.M. Glover. 2003. Mutations in makos, a Drosophila gene encoding the Cdc27 subunit of the anaphase promoting complex, enhance centrosomal defects in polo and are


The Zds proteins control entry into mitosis and target protein phosphatase 2A to the Cdc25 phosphatase. 

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Legends to the Figures

Fig. 1, Isolation of novel cdc55 alleles

(A) Sequence alignment of yeast Cdc55 and human PP2A B55. Mutations resulting in the amino acid substitution (G43D, G47R, D319N) are boxed. Tcoffee (Notredame et al., 2000) alignment program was used. Asterisks indicate conserved residues, colons indicate semi-conserved residues (B) Structure of the human PP2A holoenzyme modified from (Xu et al., 2008). The scaffold (Aα), catalytic (Cα), and regulatory B (Bα) subunits are shown in yellow, green, and blue, respectively. The positions of novel cdc55 mutations are pointed by dotted arrows. (C) Growth phenotype of cdc55 mutants. Top; cdc55-101, -102, -103 are sensitive to anti-microtubule drug benomyl. In contrast to cdc55Δ, these mutant did not exhibit cold sensitivity at 16°C and did not show abnormal morphogenesis. Bottom: Representative images of the cdc55 mutants cells grown at 16°C. (D) Benomyl sensitivity of cdc55Δ is rescued by overexpression of each catalytic catalytic subunits of PP2A-related phosphatases from 2-micron plasmids.

Fig. 2, cdc55 mutants are defective in the SAC

(A) Log phase cells of the indicated strains were diluted in YPD containing 15 μg/ml nocodazole. After 4 hours the cells were briefly sonicated and the percentage of G1, large budded (dumbell) and rebudded cells (re budding assay) was determined by microscopy (B) After 3 hours of nocodazole treatment of indicated strains, loss of sister chromosome cohesion of cen V was visualized and quantified (Straight et al., 1996) (C) Log phase cells of the indicated strains were treated with 15 μg/ml nocodazole in YPD at 25°C and the viability of the cells was measured at different time point as percentage of cells able
to form colonies on YPD medium (viability assay) (D) Serial dilutions of the strains with the indicated genotypes were spotted on YPD and on YPD containing benomyl at 24°C (E) Cultures of cells with the indicated genotypes were arrested in G1 by 0.5 μg/ml α-factor for 180 min and then release in the YPD medium containing 15 μg/ml nocodazole (t=0) and α-factor was re-added after 90 minutes to prevent cells from entering in the next cell cycle. Samples were collected at the indicated times for western blot analysis of cell extracts with anti-myc antibody. Anti-PSTAIRE was used as loading control.

**Fig. 3, cdc55-101 mutants suppress the temperature-sensitivity of cdc20-3 cells and of different APC mutants**

(A) and (B) Serial dilutions of the strains with the indicated genotypes were spotted on YPD at the indicated temperatures.

**Fig. 4, APC-Cdc20 is a target of PP2A-Cdc55 in the SAC**

Cdc55 affects mobility of Cdc16 and Cdc27 in the SDS-PAGE. Wild type and cdc55Δ cells expressing either Cdc16-3HA or Cdc27-3HA were treated with 15 μg/ml nocodazole for 2 hours. Total cellular proteins were separated by SDS-PAGE followed by Western blotting using anti-HA antibody. In the same assay, slower migration of Cdc16-HA was also observed in cdc55-101 similar to cdc55Δ. Rho1 was used as a loading control (B) Unphosphorylatable APC mutations prevent rebudding of cdc55 Δ . Cells were released in YPD medium containing 15 μ g/ml nocodazole (t=0). At the indicated time point at least 100 cells for each strain were scored to determine the percentage of G1, dumbell and rebudded cells. The
experiment has been repeat two times and it is shown just one representative data set.

(C) Unphosphorylatable APC mutations prevent sister chromatid separation of $c_{dc55} \Delta$ in nocodazole. Cells of the indicated genotypes were released in YPD medium containing 15 $\mu$g/ml nocodazole. After 3 and 5 hours, at least 150 cells of each strain were examined for sister chromatid separation. The experiment has been repeated two times and it is shown just one representative data set. Left: Representative images of sister chromatid separation after nocodazole treatment in WT and in $c_{dc55}\Delta$. Right: quantitative summary of sister separation defect.

Fig. 5, Localization of Cdc55-GFP and Cdc55-101-GFP

(A) Cdc55-GFP and Cdc55-101-GFP localization in rapidly growing culture. Nup159-mCherry was used as a marker for the nuclear envelope. 84.3 % cells showed nuclear Cdc55-GFP signal while 3.9 % cells showed Cdc55-101-GFP signal in the nucleus (n>100) (B) Western blotting confirmed that Cdc55-GFP and Cdc55-101-GFP (arrow in the figure) were expressed to a similar level. The asterisk corresponds to a non-specific band used as loading control. (C) Cdc55-GFP and Cdc55-101-GFP localization in nocodazole treated cells. 89 % cells showed nuclear Cdc55-GFP signal while 3.5 % cells showed Cdc55-101-GFP signal detected in the nucleus (n>100).

Fig. 6, Overexpression of ZDS1 rescues the temperature sensitivity of $c_{dc20-3}$ cells and causes SAC defects

(A) Serial dilutions of the strains with the indicated genotypes were spotted on SC-URA at the indicated temperatures (B) Top: Schematic representation of the Zds1 constructs:
Zds1 and Zds1Δc800. The first and the second homology region conserved in Zds1 homologues in fungal species (HR1 and HR2, respectively) and C-terminal Cdc55-binding domain (CBD; 801–913 a.a.) are depicted. Bottom: Alignment of the highly conserved CBD region of fungal Zds1 family proteins and human PP2A-B binding proteins. *S.cerevisiae* Zds1 and Zds2, *C. Glabrata* Zds1 (CAG60947), *A.gossypii* Zds1 (AEY94466), *H. Sapiens* CTCTTNBP2-NL (EAW56517) and CTNNBP2 (ABC87066).

(C) Serial dilutions of the strains with the indicated genotypes were spotted on galactose medium (YPG) and (YPG) containing 12.5 μg/ml benomyl at 24°C. (D) Effect of overexpression of *ZDS1* (*GAL1-ZDS1*) or *ZDS1Δc800* (*GAL-ZDS1Δc800*) on the SAC. Cells were first arrested by with 15 μg/ml (final concentration) of nocodazole in the raffinose medium (YPR). After 180 min in the nocodazole, either *ZDS1* or *ZDS1Δc800* was induced by galactose addition. Samples were collected at the indicate time points and at least 150 cells of each strain were examined for the presence of rebudded cells indicative of spindle checkpoint defects.. The experiment has been repeated two times and it is shown just one representative data set. (E) Western-blotting confirmed the induction of *GAL1-ZDS1* and *GAL1-ZDS1Δc800* after galactose addition in the (D).

**Fig. 7, Nuclear Cdc55 is essential for the SAC**

Serial dilutions of the cells with indicated genotypes spotted on YPD and YPD containing 15 μg/ml benomyl at 24°C (B) and (C) Cells with the indicated genotypes (all strains are deleted for *SWE1* to avoid mitotic entry delay) were first synchronized in G1 by mating pheromone and then released into medium containing 15 μg/ml nocodazole (t=0). At the indicated time points, samples were withdrawn for FACS analysis of DNA contents (B) and at each time point at least 150
cells were scored. The experiment has been repeated three times and one representative data set is shown (C).

**Fig. 8, Regulation of mitosis by PP2A-Cdc55**

PP2A-Cdc55 promotes mitotic entry in the cytoplasm. Cytoplasmic localization of PP2A-Cdc55 requires binding to Zds1/2 proteins. Nuclear PP2A-Cdc55 inhibits APC-Cdc20 when the spindle assembly checkpoint is active. Nuclear PP2A-Cdc55 also interferes with Cdc14 release and prevent mitotic exit. See discussion for more details
**Figure 1**

A. Alignment of sequences from human PP2A-B55 and S. cerevisiae Cdc55. The alignment highlights residues D319 and G43-G47.

B. Diagram showing the interaction of Bα subunit with A and C subunits.

C. Phenotypic analysis of cdc55 mutants at different temperatures and with benomyl treatment.

D. Microscopy images of WT, cdc55A, cdc55-101, cdc55-102, and cdc55-103 strains under different conditions.
Rossio Fig. 5